

REMARKS

This amendment accompanies a Request for Continued Examination. Claims 13, 14, 19-22, 25, 26, 31, 33, and 35 are pending. Claims 14, 19-22, and 26 have been withdrawn from consideration as directed to non-elected inventions. Claims 13, 14, and 19-22 have been amended. Claims 36 and 37 have been added. By this amendment, Claims 13, 14, 19-22, 25, 26, 31, 33, and 35-37 are pending.

The Provisional Double Patenting Rejection

Claims 13, 25, and 33 are provisionally rejected on the ground of non-statutory obviousness-type double patenting as then being unpatentable over Claims 41, 43-47, 57, 59, and 60 of co-pending Application No. 10/555,076. Applicants acknowledge the provisional rejection and will file a Terminal Disclaimer on an indication of allowable subject matter.

The Rejection of Claims 13 and 25 Under 35 U.S.C. § 102(b)

Claims 13 and 25 stand rejected under 35 U.S.C. § 102(b) as anticipated by Freeman et al., *Circulation* 103:357-372, January 2001 (the "Freeman reference"). Claim 13 has been amended. Claim 25 depends from Claim 13.

In the Advisory Action dated July 28, 2008, the Examiner stated that the Freeman reference teaches the mechanism of producing glucose transport, and that it does not matter how or where the instant "mechanism" is produced, "glucose uptake" has the same characteristics of "glucose transport."

As amended, Claim 13 recites a method for enhancing glucose uptake into warm-blooded animal cells including administering an effective amount of pravastatin or pharmacologically acceptable salts or esters thereof sufficient to enhance glucose uptake into warm-blooded animal cells, wherein glucose uptake does not include glucose transport.

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The Freeman reference discloses that pravastatin has been shown to effect endothelial function, and that impaired endothelial function has been shown to result in diminished capillary recruitment and, in turn, to correlate with the degree of insulin resistance. The Freeman reference concludes that "[b]y restoring endothelial function, pravastatin may significantly influence selective tissue perfusion and thereby beneficially affect glucose and insulin transport."

As was well known in the art at the time the instant application was filed, insulin increases capillary recruitment by selectively increasing perfusion (blood flow) in the microvasculature of muscle tissues, and that the increased perfusion may lead to increased access for insulin and nutrients, such as glucose, to the interstitial fluid. (See, for example, Clark, M.G., et al., "Blood flow and muscle metabolism: a focus on insulin action," *Am. J. Physiol. Endocrinol. Metab.* 284:E241-E258, 2003, attached hereto as **Exhibit A**).

Therefore, in contrast to the Examiner's assertion, it is clear that glucose transport across the capillary endothelium does not have the same characteristics as glucose uptake into cells. Because the Freeman reference does not disclose or suggest that pravastatin enhances glucose uptake into cells, the reference does not anticipate or render obvious the invention of Claim 13.

Rejoinder of Withdrawn Claims 14, 19-22, and 26

Independent Claims 14 and 19-22 are currently amended to conform to currently amended Claim 13. Rejoinder and allowance of withdrawn Claims 14, 19-22, and 26 is requested.

New Claims 36 and 37

Claim 36 is directed to a method for enhancing glucose uptake into warm-blooded animal adipocytes, comprising administering to a warm-blooded animal in need thereof an effective amount of pravastatin or pharmacologically acceptable salts or esters thereof sufficient to enhance glucose uptake into warm-blooded animal adipocytes. Support for the amendment is

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found, for example, at page 17, line 33, to page 18, line 5, of the application as filed. Claim 37 depends from Claim 36, and is directed to uptake of glucose from the interstitial fluid of peripheral adipose tissues.

Claim 36 is believed to be patentable over the Freeman reference, because the Freeman reference does not teach or suggest that pravastatin enhances glucose uptake into adipocytes.

CONCLUSION

Applicants believe that Claims 13, 14, 19-22, 25, 26, 31, 33, and 35-37 are in condition for allowance. If any issues remain that may be expeditiously addressed in a telephone interview, the Examiner is encouraged to telephone applicants' attorney at 206.695.1755.

Respectfully submitted,

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EXHIBIT A

**Michael G. Clark, Michelle G. Wallis, Eugene J. Barrett, Michelle A. Vincent,
Stephen M. Richards, Lucy H. Clerk and Stephen Rattigan**

Am J Physiol Endocrinol Metab 284:241-258, 2003. doi:10.1152/ajpendo.00408.2002

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Decreased NO signaling leads to enhanced vasoconstrictor responsiveness in skeletal muscle arterioles of the ZDF rat prior to overt diabetes and hypertension

L. A. Lesniewski, A. J. Donato, B. J. Behnke, C. R. Woodman, M. H. Laughlin, C. A. Ray and M. D. Delp

Am J Physiol Heart Circ Physiol, April 1, 2008; 294 (4): H1840-H1850.

[Abstract] [Full Text] [PDF]

Last Word on Point:Counterpoint: There is/is not capillary recruitment in active skeletal muscle during exercise

M. Clark, S. Rattigan, E. Barrett and M. Vincent

J Appl Physiol, March 1, 2008; 104 (3): 900-900.

[Full Text] [PDF]

Point:Counterpoint: There is/is not capillary recruitment in active skeletal muscle during exercise

M. G. Clark, S. Rattigan, E. J. Barrett and M. A. Vincent

J Appl Physiol, March 1, 2008; 104 (3): 889-891.

[Full Text] [PDF]

Protein Kinase C {theta} Activation Induces Insulin-Mediated Constriction of Muscle Resistance Arteries

W. Bakker, P. Sipkema, C. D.A. Stehouwer, E. H. Serne, Y. M. Smulders, V. W.M. van Hinsbergh and E. C. Eringa

Diabetes, March 1, 2008; 57 (3): 706-713.

[Abstract] [Full Text] [PDF]

Hyperinsulinemia Rapidly Increases Human Muscle Microvascular Perfusion but Fails to Increase Muscle Insulin Clearance: Evidence That a Saturable Process Mediates Muscle Insulin Uptake

E. M. Eggleston, L. A. Jahn and E. J. Barrett

Diabetes, December 1, 2007; 56 (12): 2958-2963.

[Abstract] [Full Text] [PDF]

Medline items on this article's topics can be found at <http://highwire.stanford.edu/lists/artbytopic.dtl> on the following topics:

Biochemistry .. Glucose Uptake

Medicine .. Insulin

Physiology .. Capillaries

Medicine .. Cardiovascular Disease Prevention

Medicine .. Exercise

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This information is current as of September 23, 2008 .

invited review

Blood flow and muscle metabolism: a focus on insulin action

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Clark, Michael G., Michelle G. Wallis, Eugene J. Barrett, Michelle A. Vincent, Stephen M. Richards, Lucy H. Clerk, and Stephen Rattigan. Blood flow and muscle metabolism: a focus on insulin action. *Am J Physiol Endocrinol Metab* 284: E241–E258, 2003; 10.1152/ajpendo.00408.2002.—The vascular system controls the delivery of nutrients and hormones to muscle, and a number of hormones may act to regulate muscle metabolism and contractile performance by modulating blood flow to and within muscle. This review examines evidence that insulin has major hemodynamic effects to influence muscle metabolism. Whole body, isolated hindlimb perfusion studies and experiments with cell cultures suggest that the hemodynamic effects of insulin emanate from the vasculature itself and involve nitric oxide-dependent vasodilation at large and small vessels with the purpose of increasing access for insulin and nutrients to the interstitium and muscle cells. Recently developed techniques for detecting changes in microvascular flow, specifically capillary recruitment in muscle, indicate this to be a key site for early insulin action at physiological levels in rats and humans. In the absence of increases in bulk flow to muscle, insulin may act to switch flow from nonnutritive to the nutritive route. In addition, there is accumulating evidence to suggest that insulin resistance of muscle *in vivo* in terms of impaired glucose uptake could be partly due to impaired insulin-mediated capillary recruitment. Exercise training improves insulin-mediated capillary recruitment and glucose uptake by muscle.

nutrient and hormone access; nutritive and nonnutritive flow; total muscle blood flow; muscle glucose uptake

IT IS NOW OVER SEVEN YEARS since we drew together the then rather limited information linking vascular effects to metabolism in skeletal muscle (28). The major message emerging from that article was that muscle metabolism and contractile performance were markedly affected by vasomodulators that redistributed blood flow between nutritive and nonnutritive vascular routes. In the intervening period, there have been some notable developments in this field based largely on new techniques for measuring microvascular flow changes and particularly insulin's action to increase microvas-

cular (nutritive) perfusion of muscle. It is thus timely to review these and related advances. This review also affords an opportunity to examine a significant previous omission concerning the effects of bulk flow change, particularly that evoked by insulin, on muscle metabolism.

INSULIN-MEDIATED INCREASES IN BULK BLOOD FLOW TO SKELETAL MUSCLE

Insulin-mediated vasodilation. Interest in this area was renewed in the 1990s by Baron and colleagues [Laakso et al. (93)], when they reported insulin's ability to increase total blood flow to skeletal muscle and suggested that this may, in fact, enhance insulin action by augmenting the delivery of insulin and glucose to

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Table 1. Comparison of insulin effects on muscle capillary recruitment, total blood flow, and glucose uptake

Parameter	Capillary Recruitment	Total Blood Flow	Muscle Glucose Uptake
Timing	Early, 5–10 min in rats (185) Not yet examined in humans	90–120 min in rats (186) Controversial issue: implied to coincide with glucose uptake in human leg muscle [(9) and references therein]; may only coincide when doses are supraphysiological (183)	>30 min in rats (185) <60 min at physiological insulin [e.g., 60 mU/l in humans, (183)]; see also (9).
Dose	Increased at 100 mU/l in rats (186)	Increased at 100 mU/l in rats (186)	Half maximal at 80–320 mU/l in rats dependent on muscle type (82)
	Increased at 55 mU/l in human forearm (34)	No change at 55 mU/l in human forearm (34) Increased at physiological levels in humans, but extended times may be required (2) (93,190)	Increased at 55 mU/l in human forearm (34) Leg glucose uptake half maximal at 70 mU/l (94)
Mechanism	No dependent in rats (189)	NO dependent in rats (189) and humans (148) and (9,165)	Partially NO dependent in rats (189) and humans (8) Partly increased by vasodilator methacholine (147,165)
	Masked accompanying vasoconstriction (140)	Masked accompanying vasoconstriction possibly mediated by endothelin-1 (22)	
Proposed sites	Terminal arterioles	Feed arteries	Skeletal muscle
Correlations	With muscle glucose uptake in rats (137) Correlation with whole body glucose uptake in rats not tested	Relationship to glucose disposal in humans strong (102) or non-existent (198)	With capillary recruitment in rats (137) Correlation of leg blood flow and leg glucose uptake in humans (102) Correlation of whole body glucose uptake and leg blood flow in humans (9)
Exercise	Increased in dog (69) and rats (39)	Increased but at higher frequency stimulation than capillary recruitment (69)	Increased [(142) and references therein]
Impairment	Obese human forearm (34)	Obese human leg (93) NIDDM human leg (94) Hypertensive patients [(164) and references therein] Free fatty acids impair NO-dependent mechanism (9)	Obese human leg (93) NIDDM human leg (94)
	Zucker obese rats (26)	Zucker obese rats (26)	Zucker obese rats, partly impaired (26)
	Acute TNF- α -treated rats (201)	Acute-TNF- α -treated rats (201)	Acute TNF- α -treated rats, partly impaired (201)
	Acute Intralipid-heparin-treated rats (31)		Acute Intralipid-heparin-treated rats, partly impaired (31)
	Acute pharmacological hypertension in rats (137)	Acute pharmacological hypertension in rats (137)	Acute pharmacological hypertension in rats, partly impaired (137)
Enhancement	Voluntary exercise training in rats (139)	No effect of voluntary exercise training in rats (139)	Voluntary exercise training in rats (139)

NIDDM, non-insulin-dependent (type 2) diabetes mellitus; NO, nitric oxide.

muscle cells. Those studies were carried out in human subjects during a hyperinsulinemic euglycemic clamp to minimize the possible effects of counterregulatory hormones. A number of investigators have repeated these findings in both humans (2, 40, 133, 170, 180, 183, 190) and animals (101, 140); however, there is considerable variation between studies in the magnitude of the response, and in fact some groups have failed to observe changes in flow with insulin (46, 47, 80, 88, 145, 199) (see also Table 1). The discrepancies are not related to the method used to determine flow, since changes have been detected by thermodilution (40, 93), dye dilution (51), plethysmography (2, 180, 183, 190), positron emission

tomography (PET) combined with [^{15}O]H $_2$ O (133), and ultrasound in animals (136). Systemic insulin (2, 40, 170, 183, 190) vs. local intra-arterial infusion (51, 116, 171, 180) also does not seem to explain the difference, as vasodilation has been reported in both situations. Interestingly, Ueda et al. (180) did report greater increases in flow when the local intra-arterial infusions in the forearm were combined with an infusion of a physiological concentration of glucose. Nevertheless, the magnitude of vasodilation was similar in forearm and calf during systemic insulin infusion (183).

What does appear to be important was subject selection, since there was a large interindividual variation

in response, which has been attributed to the level of physical activity, muscularity, and capillarization (181, 182). Also, results may differ depending on the protocol used, as the increase in total flow was more apparent at higher insulin concentrations and after longer exposure times (198). This raises the question whether the increases in flow are physiologically relevant. Although many studies do use supraphysiological doses of insulin (11, 16, 133), there are also reports of dilation at physiological doses (2, 93, 190) (Table 1). However, some of these studies have been criticized (183) because sequential insulin infusions were used, so that, although the doses were physiological, the infusion had often been maintained for several hours before an increase in flow was detected (93). There are contradictory reports regarding the dose curve for insulin action on glucose uptake and flow. Laakso et al. (94) reported similar half-maximal concentrations for leg glucose uptake and flow (420 and 266 pM, respectively). Yet Utriainen et al. (183) found that, at low insulin concentrations (366 pM, 60 mU/l), glucose extraction increased maximally before the small increase in flow (17%). With higher insulin levels (2,772 pM), the arteriovenous glucose concentration difference did not increase further; however, the glucose uptake continued to rise further as a result of the increased flow (113%). Thus they concluded that flow becomes an important modulator of glucose uptake only at supra-physiological concentrations of insulin (183). Similarly, others have also reported that glucose uptake rises before total blood flow (50, 171) (see also Table 1).

Although the hyperinsulinemic euglycemic clamp is a commonly used technique that has many advantages for scientific research, it is unphysiological in that high insulin levels are not normally sustained for long periods after a meal. Despite this, an increase in skeletal muscle blood flow has been demonstrated following both intravenous glucose (99, 190) and oral glucose load (13) and a carbohydrate meal (152), which more closely mimics the physiological situation. However, there was no significant rise in leg blood flow after a mixed meal (64). Similar reasoning has recently been applied in animal studies where a bolus injection of insulin caused vasodilation but a bolus of glucose that increased insulin release resulted in a vasoconstriction of the muscle vasculature (140).

To summarize, it is generally accepted that, in experimental situations, insulin does increase total flow to skeletal muscle, and there is increasing evidence that insulin stimulates total flow under physiological situations. However, it is still uncertain whether the increase in total flow impacts on muscle glucose uptake by enhancing access for hormone and substrate (Table 1). This will be discussed further in INSULIN-MEDIATED CAPILLARY RECRUITMENT.

Vascular resistance and cardiac output. Whereas insulin augments skeletal muscle flow, it does so with either no change (134) or a small decrease in mean arterial pressure (MAP) (10, 183). Accordingly, there is a fall in leg or forearm vascular resistance, calculated from MAP divided by limb blood flow (10, 134).

For flow to be increased to skeletal muscle, there must be either an increase in cardiac output or a redistribution of flow between organs. There are reports of increased cardiac output both following a mixed meal (4) and during a hyperinsulinemic euglycemic clamp (10). At plasma insulin levels of 212 pM, cardiac output was not significantly changed, whereas at 468 and 12,872 pM there was an increase in cardiac output with a concomitant increase in heart rate and stroke volume. Systemic vascular resistance is calculated from MAP divided by cardiac output. Therefore, insulin infusion was associated with a fall in systemic vascular resistance (10). This fall in systemic vascular resistance was less than that for leg vascular resistance, indicating that insulin preferentially vasodilates in skeletal muscle. Hence, it is reasonable to conclude that insulin redistributes increased cardiac output to skeletal muscle.

Sympathetic nervous system activation. There are several reports that insulin increases sympathetic nervous system (SNS) activity, as assessed by venous catecholamine levels (144) and forearm norepinephrine (NE) release (98) as well as by direct measurements of muscle sympathetic nerve activity (MSNA) (2, 190). These sympathoexcitatory effects of insulin appear to be centrally mediated, since they are apparent only during systemic insulin infusion but not following local infusion (98). As mentioned previously, during a hyperinsulinemic euglycemic clamp, there is generally no change in MAP. This is possibly due to the opposing effects of increased sympathetic vasoconstrictor action and a decrease in skeletal muscle vascular resistance (190). However, in patients with autonomic failure, a decrease in blood pressure is seen, since there is no sympathetic pressor effect (103). Although it was suggested that MSNA may be stimulated as a baroreflex to maintain blood pressure following vasodilation, this does not seem to be the case, since Spraul et al. (162) reported that MSNA increased before total flow and there was no correlation between the two. They also concluded that the increased MSNA did not cause the increased flow through sympathetic vasodilation. This is supported by the findings of Randin et al. (134) that insulin-mediated vasodilation was unaltered by the cholinergic blocker atropine and the β -adrenergic blocker propranolol. Moreover, from experiments on patients with complete motoric lesion of the cervical spinal cord, Dela et al. (42) demonstrated that the central nervous system is not involved in insulin-mediated vasodilation, as the percentage of increase in leg blood flow was similar to that of healthy individuals. Comparisons of absolute increases in flow were complicated by the lower basal flows in the patients with spinal cord injury that are likely to be a result of decreased muscle mass, although this was not measured.

INSULIN-MEDIATED CAPILLARY RECRUITMENT

Although most studies have focused on increases in total flow to skeletal muscle, what may be more impor-

tant is the distribution of flow within this tissue. Before this is discussed, it is necessary to give a brief description of the structure of the skeletal muscle vasculature and the possibility of two flow routes in this tissue.

Structure of skeletal muscle vasculature. Not all muscles are suitable for viewing the microvasculature *in situ*. Therefore, most studies have been performed on muscles such as the cremastor, hamster retractor, tenuissimus, or spinotrapezius muscle because of the thinness of the muscle and the ease in which vessels can be viewed. There do appear to be similarities in the structure of the microvasculature between different muscles; however, comparisons must be made with caution, because these are often very specialized muscles and may differ considerably from the structure found in load-bearing cylindrical muscles (112).

The arrangement of arterioles in the hamster cremastor muscle has been described by Sweeney and Sarelius (169). Moreover, they have used this preparation to investigate which vessels control flow distribution during hyperemia. Blood enters the muscle from the feed artery, which is generally classified as a first-order arteriole. Subsequent branches are numbered in increasing order, with transverse arterioles being third order and fifth-order arterioles leading to capillaries (169). The capillaries in muscle are arranged in groups of ~15, which arise from one fifth-order arteriole and are classified as one module (111). The group of modules arising from one fourth-order arteriole is termed a capillary network (111).

Flow to the whole muscle is controlled by the first- to third-order arterioles, whereas flow to individual capillary networks and, hence, the flow distribution within muscle is controlled by the third- to -fifth-order arterioles (111). At any one time, not all capillaries in resting muscle are perfused (70, 169). It has been proposed that blood vessels undergo vasomotion, contracting at regular intervals to alternately direct blood flow through different capillary modules (76).

Evidence for two vascular flow routes in muscle. Work done in our laboratory using the perfused rat hindlimb supports earlier proposals by others that there are two flow routes in muscle: one in intimate contact with the myocytes and able to exchange nutrients and hormones freely and thus regarded as nutritive, and a second with essentially no contact with myocytes and regarded as nonnutritive [the history and background of this concept together with references can be found elsewhere (28)] (29, 30). It is thought that the nutritive network consists of long, tortuous capillaries, which have large surface area for optimal exchange with the muscle cells. On the other hand, the nonnutritive network may be made up of shorter, possibly slightly larger capillaries, as judged by passage or failure of passage of different-size microspheres (188) of lower resistance. Some of the nonnutritive route may supply muscle connective tissue (septa and tendon) (121) and nourish associated adipocytes (32). Laser Doppler flowmetry (LDF) using impaled micropipes (200- μ m diameter) indicates that the nonnutritive ves-

sels may be distributed evenly throughout the load-bearing muscles but are relatively fewer than nutritive vessels (27). The data are consistent with the proposition that these nonnutritive vessels carry a flow reserve, which can redistribute to the nutritive route during periods of high metabolic demand, such as during exercise (30) or after a meal, to allow insulin-mediated glucose uptake into muscle. The balance between these two circuits is controlled by vasomodulators and neural input. In the constant flow pump-perfused hindlimb, we have classified vasoconstrictors according to whether they increase or decrease metabolism (type A and type B, respectively) (28). Examples of type A vasoconstrictors include low-dose NE, angiotensin II, vasopressin, and low-frequency SNS activation. These all redirect flow and increase oxygen consumption. Serotonin, high-dose NE, and high-frequency SNS activation are all type B vasoconstrictors, and these decrease oxygen consumption and metabolism generally (28). Changes in flow redistribution are suggested from vascular casts and changes to the pattern of red cell washout (119). There is evidence that oxygen uptake by resting muscle is a function of oxygen delivery (92), thus offering an explanation why the vasomotion-mediated redistribution of flow within muscle has such profound effects on oxygen uptake. During type B vasoconstrictor action, a large proportion of the muscle very likely undergoes a physiological hypoxia. As indicated above, the constant vasomotion activity may allow the sharing of this over the entire muscle with time. Evidence to date shows that only the phosphocreatine-to-creatine ratio is compromised by a low ratio of nutritive to nonnutritive flow, and this is reversed when the nutritive-to-nonnutritive ratio is increased (187). For present considerations, the presence of this nonnutritive route in muscle may explain two phenomena that are developed further in sections below. These are 1) the ability of many systemic vasodilators to markedly increase limb blood flow without affecting insulin-mediated glucose uptake (114, 123) or ameliorating insulin resistance (115), and 2) the ability of insulin to increase capillary recruitment without either an increase in limb blood flow or a slowing in mean cell velocity in the capillaries (186). Other workers in the past have also invoked the notion of a nonnutritive route or functional shunt to explain the differences in clearance of intramuscular injected or infused markers due to exercise or vasodilators (see Ref. 30 and references therein).

Capillary recruitment: technical approaches for determining capillary recruitment in vivo. In an indirect approach, Bonadonna et al. (16) utilized a pulse injection into the brachial artery of L-[1- 3 H]glucose, an extracellular marker, that fills both blood vessels and the interstitial space. By analyzing the washout curve, the amount of tissue drained by the deep forearm vein was estimated. Although there was no change in the amount of muscle tissue drained when saline and a physiological dose of insulin (400 pM) were compared, there was an increase in the extracellular volume in response to supraphysiological levels of insulin (5,600

pM). This was interpreted by the authors to indicate capillary recruitment.

Another method to indirectly assess capillary flow is by observation of the microvasculature in situ using intravital microscopy. It was demonstrated in the spinotrapezius muscle by Renaudin et al. (140) that a subcutaneous injection of insulin to anesthetized rats dilated precapillary arterioles but did not alter vasomotion, whereas following glucose infusion to obtain similar insulin levels there was an increased vasomotion, despite a small constriction. The authors proposed that this would lead to a more optimal perfusion of the microvasculature. Indeed, as pointed out by Wiernsperger (193), a vasoconstrictor activity of insulin that is masked by a larger vasodilatory effect may be essential to the normal functioning of this hormone.

We have recently reported an insulin-mediated increase in capillary recruitment in muscle both in animals (136) and in humans (35) in vivo. This represented the first report of a direct effect of insulin and was the result of our experience with perfused muscle preparations, the realization of two vascular routes in muscle, and the development of specific techniques for the assessment of changes in flow in one of these routes (i.e., nutritive or capillary flow). The first of these techniques is based on the metabolism of 1-methylxanthine (1-MX), an exogenous substrate for xanthine oxidase. Immunohistochemical evidence shows that xanthine oxidase is concentrated in the muscle capillaries and is much less expressed in larger vessels and muscle itself (61, 83). Our studies have shown that changes in 1-MX metabolism correlate positively with changes in the proportion of nutritive flow in pump-perfused muscle under a number of conditions (135, 200). More importantly, when applied to anesthetized, ad libitum-fed rats in vivo, we found that insulin (at doses that increased insulin levels 4-fold) increased 1-MX metabolism (136) in association with increased glucose uptake of hindlimb muscle. The changes in 1-MX metabolism are indicative of capillary recruitment that can be considered nutritive to the muscle cell. Although the insulin-mediated increase in capillary recruitment was accompanied by an increase in femoral artery blood flow, epinephrine infusions that produced similar increases in femoral artery blood flow did not affect capillary recruitment (measured by 1-MX extraction), indicating that the two processes can be dissociated. This is a crucial observation, as it demonstrates that a vasodilator such as epinephrine can increase total leg blood flow in vivo without increasing capillary recruitment or glucose uptake. Again, this dissociation between total flow and capillary recruitment may help to explain the human data where a number of vasodilators similarly increase flow but differentially affect glucose uptake. As alluded to above, the total flow increase in these circumstances may be carried by the nonnutritive route. Another important point is that the increase in 1-MX metabolism with insulin preceded the increase in total blood flow. To carry this notion to the next stage, we have shown that a vasoconstrictor (α -methylserotonin) that decreases

nutritive flow in perfused muscle (117) inhibits insulin-stimulated hindlimb 1-MX extraction in vivo and impairs insulin-mediated glucose uptake in the same hindlimb muscles (137). Moreover, TNF- α , which is elevated in various insulin-resistant states, completely blocks the hemodynamic actions of insulin, including the increase in 1-MX metabolism and ~50% of the insulin-mediated glucose uptake (201). Overall, insulin-stimulated glucose uptake by the hindleg in vivo correlates tightly with 1-MX metabolism, an indicator of capillary recruitment, but shows no significant correlation with total limb flow (137) that was measured in these same studies. It would seem likely that this vascular action of insulin enhances perfusion of muscle, independently of changes in total flow, particularly in a time perspective. If this proves to be so, then insulin-mediated redistribution of blood flow from the nonnutritive route is likely.

The second technique that we have adapted to provide information on capillary perfusion in skeletal muscle is contrast-enhanced ultrasound (CEU) imaging. The technique is based on that described by Wei et al. (192) for heart, in which microbubbles of albumin provide the contrast medium. A prototype Sonos 2500 system (Hewlett-Packard) collects transverse section images in harmonic mode, in which ultrasound is transmitted at 2 MHz and is received at 4 MHz. Ultrasound pulses are gated to an internal timer to allow variable time intervals between pulses. The rationale is to destroy the microbubbles during the first ultrasound pulse and image their reappearance within the muscle vasculature with the second pulse. During an imaging sequence, the interval between these pulses is progressively prolonged to allow more replenishment of the ultrasound beam elevation. Once the images are collected, a background subtraction is made to isolate effects on capillaries. The time rate of appearance of the microbubbles, which have no effect on systemic hemodynamics, allows an estimate of the microvascular volume and the average filling velocity. By use of this approach, changes in capillary blood volume in response to insulin and exercise have recently been assessed in the skeletal muscle of the rat hindlimb in vivo and compared with data obtained using 1-MX metabolism (39). Compared with baseline values, saline infusion resulted in little change in capillary blood volume, whereas marked increases in capillary blood volume occurred during a euglycemic insulin clamp ($3 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) or exercise produced by hindleg electrostimulation. CEU data correlate well with 1-MX metabolism data, and capillary blood volume increases two- to threefold during these physiological doses of insulin (39). There is no change in mean cell velocity even at the end of a 2-h clamp (39), and the increase in bulk blood flow due to physiological insulin was not apparent in the 1st h (186), again pointing to the likelihood of an insulin-mediated redistribution of blood flow to account for the capillary recruitment. At this stage, we would propose that microbubble filling of the shorter capillaries of the nonnutritive route is rapid and may be removed in the image subtraction

step intended to remove larger, rapidly filling vessels. Most recently, time course studies have shown that capillary recruitment by insulin occurs within 5–10 min and thus may be one of the earliest events of insulin action in vivo (185).

The third approach is LDF, a technique that has been used for a number of years to study skin blood flow. Recently, several research groups have documented impaired skin microvascular reactivity in diabetic subjects (19, 79, 155). In such studies, microvascular function was assessed by measuring vasodilation in the forearm skin in response to iontophoresis of acetylcholine (79, 155), although hyperemic response to local heating (19) was also used. Most significantly, Serne et al. (154) have recently shown that iontophoretically administered insulin directly vasodilates skin microvascular blood flow. In our own studies using LDF, we have found that the signal strength from relatively large probes (800 μm), when measured over muscle, directly related to the extent of nutritive flow in the constant flow-perfused rat hindlimb (25). Thus vasoconstrictors that increase metabolism in this preparation increase the LDF signal; conversely, vasoconstrictors that decrease metabolism also decrease LDF signal (25). LDF probe size appears to be particularly important. For example, when smaller probes (200 μm) are randomly impaled into muscle, heterogeneity of sites is detected, with ~60% showing an increase in LDF signal with stimulatory type A vasoconstrictors, 15% showing a decrease, and 25% showing no alteration in signal (27). These same sites responded in the opposite manner to inhibitory type B vasoconstrictors with ~60% showing a decrease in LDF signal, etc. Overall, the data from the LDF studies lend strength to the proposal of two vascular networks in skeletal muscle, nutritive and nonnutritive. The smaller LDF probes allow resolution at the level of individual networks (either nutritive or nonnutritive), and the larger probes essentially present an average, which, since the nutritive outweighs the nonnutritive by at least 3:1, is largely representative of nutritive flow. Importantly, when the larger probes were applied to rats under the euglycemic hyperinsulinemic clamp in vivo, the LDF signal increased, coincident with insulin-mediated increases in glucose infusion (25). The time course for insulin effects in vivo showed that the LDF signal increased well before a change in femoral arterial blood flow (25).

This suggests that LDF is sensitive to a flow component that is not total flow but is likely flow in the capillaries of the nutritive network. This would appear to confirm findings with 1-MX that insulin mediates a marked capillary recruitment in muscle as part of its action in vivo. If capillary nutritive flow occurs independently of changes in total flow, as is suggested, then flow must be switched by the action of insulin from the nonnutritive route.

Data from the application of each of these techniques during the hyperinsulinemic euglycemic clamp are summarized in Table 2. Insulin significantly increased femoral arterial blood flow and hindleg glucose uptake, as well as the indicators of capillary recruitment, hindleg 1-MX metabolism, surface LDF signal, and CEU. Epinephrine, which increased femoral arterial blood flow, did not increase glucose uptake, 1-MX metabolism, or LDF signal and would thus be likely to have increased nonnutritive flow. Exercise increased both 1-MX metabolism and CEU. Taken together, the data support the view that each of the techniques is measuring capillary recruitment and that this is increased by insulin or exercise.

MECHANISMS FOR THE VASCULAR ACTIONS OF INSULIN

Insulin effects in cultured cells and isolated vessels. To better understand the mechanisms of insulin-mediated increases in both total flow and capillary recruitment, it is useful to consider insulin's action in isolated vessels and cultured cells. However, studies in these in vitro systems must be interpreted with caution, because the conditions do not necessarily mimic those in vivo and insulin's actions appear to vary in different vascular beds. Also, because supraphysiological doses of insulin are often used in vitro, it is possible that the effects seen are due to insulin's interaction with insulin-like growth factor I (IGF-I) receptor rather than the insulin receptor (65). Nevertheless, these studies are valuable because they indicate insulin's effects on specific cell types in the absence of systemic effects.

High concentrations of insulin (600, 1,500, and 2,400 pM) have been reported to attenuate the vasoconstrictor effects of NE, serotonin, and potassium chloride in mesenteric arterioles (191). In contrast, Wu et al. (195) found that insulin (60 nM) potentiated the vasocon-

Table 2. Effects of insulin, epinephrine, exercise, or saline on FBF, glucose uptake, and 3 putative indicators of muscle nutritive flow: 1-MX metabolism, surface LDF, and CEU microbubbles

Condition	FBF, ml/min	Glucose Uptake, $\mu\text{mol/min}$	1-MX, nmol/min	LDF, V	CEU (video intensity)
Saline	0.81 ± 0.04	0.26 ± 0.04	5.7 ± 0.5	0.27 ± 0.06	6.8 ± 0.9
Insulin	$1.45 \pm 0.21^*$	$1.05 \pm 0.15^*$	$8.6 \pm 1.3^*$	$0.45 \pm 0.05^*$	$10.1 \pm 1.1^{*\dagger}$
Epinephrine	$1.83 \pm 0.42^*$	0.31 ± 0.06	5.5 ± 0.5	0.30 ± 0.07	
Exercise	$2.73 \pm 0.17^*$		$11.0 \pm 2.2^*$		$13.3 \pm 2.0^*$

Values are means \pm SE. FBF, femoral arterial blood flow; 1-MX, 1-methylxanthine; LDF, laser Doppler flowmetry; CEU, contrast-enhanced ultrasound. Insulin was infused at either 10 or 3 $\text{mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ under hyperinsulinemic euglycemic clamp conditions. Data for saline, insulin at 10 $\text{mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, and epinephrine are from (136). Data for saline, insulin at 3 $\text{mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, and exercise are from (39). *Significantly different from corresponding Saline value.

striction due to arginine vasopressin in the perfused mesenteric artery. This is in agreement with the findings of Baron and Brechtel (10) that, during insulin clamps, systemic vascular resistance fell less than leg vascular resistance, indicating vasodilation in the leg, with possibly a small vasoconstriction in other vascular beds. Indeed, there have been reports that insulin does dilate isolated vessels from skeletal muscle. Schroeder et al. (150) examined first-order arterioles isolated from red and white gastrocnemius muscle. They found that, within the physiological range (60 and 600 pM), insulin increased vessel diameter; however, this effect was not seen following endothelium removal or after incubation with the nitric oxide synthase (NOS) inhibitor N^G -nitro-L-arginine (L-NNA). In fact, a significant vasoconstriction was seen with L-NNA treatment. On the other hand, prostaglandins did not appear to be involved, as vasodilation was not altered by indomethacin.

Studies in cultured human umbilical vein endothelial cells (HUVEC) also demonstrate that insulin increases L-arginine transport (160) and stimulates the production of NO (204). Furthermore, this process was shown to involve the stimulation of phosphatidylinositol (PI) 3-kinase and Akt (108, 203, 204). However, it is also possible that NO is produced from vascular smooth muscle. Indeed, Trovati and colleagues (178, 179) reported increases in cGMP in human vascular smooth muscles following incubation with insulin (240–960 pM). This effect was blocked by the NOS inhibitor N^G -monomethyl-L-arginine (L-NMMA) and methylene blue (a guanylate cyclase inhibitor), indicating that insulin stimulates the production of NO in vascular smooth muscle cells (VSMC), which, in turn, activates guanylate cyclase, leading to increases in cGMP. Although Kahn et al. (85) did not see increases in cGMP in cultured VSMC incubated with insulin (1 nM) alone, in the presence of serotonin, insulin did increase cGMP levels. This was associated with an inhibition of serotonin-induced contractions and involved the production of NO.

In addition, insulin is known to activate the Na^+ - K^+ -ATPase in VSMC, possibly by stimulating translocation of the Na^+ - K^+ -ATPase to the plasma membrane (75, 126) or through stimulation of Na^+ - H^+ exchange (109). Increased Na^+ - K^+ -ATPase activity will result in hyperpolarization of the cell, blocking of voltage-dependent calcium channels, and relaxation (86). Furthermore, insulin also decreases intracellular calcium levels by enhancing calcium efflux through activation of the Ca^{2+} -ATPase (202) and by inhibiting calcium influx controlled by both voltage- and receptor-operated calcium channels (reviewed in Ref. 110), both of which lead to VSMC relaxation.

Thus there is more than circumstantial evidence that the hemodynamic effects of insulin noted in vivo occur in vitro with cultured cells and vessels of the vascular system. Insulin attenuates vasoconstrictor activity and dilates arterioles of skeletal muscle in an endothelium-dependent manner, and both endothelial

cells and vascular smooth muscle respond directly to insulin, consistent with an outcome of vasorelaxation.

Mechanisms of insulin-mediated increases in bulk flow: in vivo evidence. Increases in blood flow in response to insulin could result from either a direct vasodilator action of insulin via receptors on the vascular cells or indirectly from a signal generated from the stimulation of metabolism within skeletal muscle cells. The latter possibility is analogous to exercise-induced hyperemia, where muscle blood flow increases to provide sufficient oxygen and nutrients to support muscle contraction. Although the exact identities of the metabolic vasodilators have not been pinned down, there is evidence for the involvement of adenosine, potassium, NO, and/or lactate (153). It is conceivable that any one of these could mediate the increased flow in response to insulin. McKay and Hester (105) found evidence of a role for adenosine in the response to insulin (1,200 pM) applied topically to cremaster muscle of anesthetized hamsters. Dilation of second- and fourth-order arterioles by insulin was blocked by the adenosine receptor antagonist 1,3-dipropyl-8-(4-sulphophenyl)xanthine. They showed that the dilation was affected by opening of ATP-sensitive K^+ channels, which have been implicated in adenosine-mediated vasodilation. Conversely, studies by Vollenweider et al. (190) have suggested that increased muscle cell glucose metabolism, to generate a putative metabolic vasodilator, fails to cause vasodilation in the absence of insulin. Whereas combined insulin and glucose infusion significantly increased calf blood flow, there was no such increase during fructose infusion in which insulin levels remained low, but a similar rise in carbohydrate oxidation was achieved. However, as pointed out by Baron (7), although the rates of whole body carbohydrate oxidation were equivalent, this is not necessarily true for skeletal muscle, as fructose oxidation may have taken place in other tissues.

Studies by Messina and colleagues [Chen and Messina (23) and Schroeder et al. (150)] point to a vasodilation induced by insulin itself, acting directly on blood vessels, rather than a metabolic vasodilation. Isolated first-order arterioles (77 μm resting diameter) from red or white gastrocnemius dilated to insulin concentrations as low as 60–600 pM (9–13% increase in diameter). This dilation involved NO generated by the endothelial cells, as demonstrated by endothelial removal or inhibition by the NOS blocker L-NNA, but was not affected by the prostaglandin inhibitor indomethacin (150). Indeed, insulin has been shown to directly activate a signaling cascade leading to endothelial (e)NOS phosphorylation in cultured endothelial cells via insulin receptor substrate (IRS)-1, PI 3-kinase, and PKB/Akt (89). Results from in vitro experiments indicating an important role for NO in the increase in total flow are supported by in vivo experiments. During a hyperinsulinemic euglycemic clamp in humans, the NOS inhibitor L-NMMA was shown to block insulin-mediated increases in total blood flow (148, 165).

Insulin's ability to enhance NO production may also be important in the modulation of vascular responses, since Lembo et al. (97) demonstrated that there is an NO component to the α_2 - and β -adrenergic responses. Moreover, they showed that insulin is able to enhance this NO component, resulting in blunted vasoconstriction due to the α_2 -agonist BHT-933 and enhanced dilation in response to the β -agonist isoproterenol.

In summary, evidence favors an NO-dependent mechanism by which insulin controls bulk flow, and many studies support a direct interaction of insulin with insulin receptors on endothelial cells. There is growing evidence for a link from insulin receptor to NOS activation via IRS-1, PI 3-kinase, and PKB/Akt.

Mechanism of capillary recruitment. At present, the mechanism of insulin-mediated capillary recruitment remains elusive. Although insulin's action to increase total flow is likely to involve first- and second-order arterioles, the capillary recruitment is probably mediated by third- to fifth-order arterioles. Certainly, small arterioles respond to insulin, either systemically or topically applied to the muscle. Iwashita et al. (78) observed dilation of fourth-order arterioles (10 μ m diameter), viewed by intravital microscopy of rat cremaster muscle in response to subcutaneous insulin injection resulting in a serum insulin of 835 pM. Vasodilation resulting from epinephrine release due to the fall in blood glucose (from 6.7 to 5.8 mM) in these experiments cannot be ruled out, however. Porter et al. (131) established that third-order arterioles (20 μ m diameter) were much more responsive to insulin than larger vessels in cremaster muscle *in situ*, albeit at very high concentrations of topically applied insulin (48 nM). A similar increase in sensitivity to insulin-mediated vasodilation with decreasing vessel size has been reported elsewhere (105, 125).

Although insulin appears to dilate isolated larger vessels directly, it is not known whether the mechanisms of vasodilation in small arterioles are similar. The inaccessibility of third- and fourth-order arterioles to isolation means that direct actions of insulin have not been studied. As discussed above for increases in total flow, it is possible that insulin may have either a direct or an indirect action. Insulin receptors are present on endothelial, vascular smooth muscle, and skeletal muscle cells. Thus insulin may have a direct action on any of these cell types to activate the insulin-signaling cascade and either directly cause VSMC relaxation or lead to the release of a vasodilator. Alternatively, it may be the glucose uptake by skeletal muscle cells stimulated by insulin that is responsible for the formation of a vasodilator. It is equally possible that it is the glucose metabolism in VSMC that is important, since they also express GLUT4 (6). In either situation, the identity of the vasodilator is unknown, but recent evidence favors the possibility that NO is involved. The vasodilator is expected to act locally, diffusing from the site of formation to the VSMC of the terminal arterioles. If, as current data suggest, the capillary recruitment does occur before the increase in total flow, it is possible that flow is redistributed from

so-called nonnutritive vessels to nutritive capillaries. The dilation of small arterioles in response to topical insulin in muscle preparations *in situ* (78) precedes changes in muscle cell glucose uptake, and this is consistent with our own recent observations of insulin-mediated capillary recruitment *in vivo* as soon as 5 min after insulin infusion (185). This is despite reports (78, 131) of dilation of third- and fourth-order arterioles only after 15 min of insulin treatment. However, there are some studies reporting the direct dilation by insulin of isolated first-order gastrocnemius arterioles requiring 3 min to observe an effect, and 5–11 min to reach maximal dilation (150). Such time frames favor the notion of a direct vascular effect of insulin involving activation of a signaling cascade leading to phosphorylation and activation of NOS. Inhibition of capillary recruitment by a NOS inhibitor in rats during a euglycemic hyperinsulinemic clamp has recently provided some support for a direct dilatory action of insulin on small vessels of muscle (189), as does a recent report by Serne et al. (154) in humans of a direct effect of iontophoresed insulin on dilatation of skin microvessels *in vivo*, with matching vasodilatory effects in response to systemically applied insulin. Such findings may explain, therefore, why the eNOS knockout mouse exhibits a degree of muscle insulin resistance (45, 156). However, these results from the eNOS knockout mouse must be interpreted with caution, as similar muscle insulin resistance in neuronal (n)NOS knockout mice (156) suggests that skeletal muscle cells, rather than endothelial cells, may be the source of the NO production stimulated by insulin.

When the *in vitro* data are considered, it seems that insulin can act on endothelial, vascular smooth muscle, and skeletal muscle cells. Thus it is quite possible that the capillary recruitment is the net result of a combination of effects on these different cell types. In other words, there may not be one single mechanism. Hence, modulation of any of these actions could potentially alter the overall recruitment.

INFLUENCE OF BLOOD FLOW ON GLUCOSE UPTAKE

Whereas it is generally accepted that insulin can increase total blood flow, controversy still remains as to the physiological relevance of this, both in regulating normal glucose uptake and as a possible cause of insulin resistance. The notion that insulin-mediated glucose uptake is improved by increasing hormone and substrate delivery implies that flow is rate limiting, either in the bulk sense or at the level of the microvasculature.

Early studies using the perfused rat hindlimb showed that increasing glucose delivery with or without insulin raises glucose uptake (55, 151). Grubb and Snarr (55) demonstrated that increasing glucose concentration within the physiological range increased glucose uptake in a linear fashion. However, with a constant glucose level, the relationship between flow and glucose uptake was hyperbolic. They proposed that this saturation of glucose uptake might be due to flow

being shunted into a parallel network with low nutrient exchange. Importantly, though, at physiological flow rates, glucose uptake was not maximal; thus further increases in flow would be expected to increase glucose uptake.

Limb glucose uptake can be calculated by using the Fick principle, which states that glucose uptake is equal to the product of the arteriovenous glucose difference and flow (205). Therefore, increasing either flow or cellular permeability to glucose will increase glucose uptake. When a simplified model of a single capillary of fixed surface area is considered, glucose uptake is limited by either permeability or flow. If the capillary is completely permeable to glucose, then, depending on metabolism, the extraction will be 100% and thus flow could become rate limiting; however, in a situation in which the extraction is low, an increase in flow will have no effect on the rate of glucose uptake (141). It has been reported that, in vivo, ~40% of glucose is extracted, so that, in fact, the situation is a combination of the two scenarios (7). As glucose is extracted from the capillary, its concentration drops, forming a gradient from the arterial to the venous end of the capillary. By increasing flow, this gradient is reduced, although it was estimated that, in vivo, this would increase glucose uptake only ~10% (7). However, if capillary recruitment also takes place, the capillary surface area will be increased, further enhancing glucose uptake.

To investigate this issue, Natali (113) compared experimental data with a model of gradient dilution and capillary recruitment. In the capillary recruitment model, as flow was increased, glucose uptake would be expected to increase in a linear fashion, whereas in the gradient dilution scenario increasing flow would cause a hyperbolic increase in glucose uptake. In the experiments (113), flow was altered during a hyperinsulinemic euglycemic clamp in healthy humans. It was found that the data fitted a model of capillary recruitment when flow was reduced by ouabain and fitted a model of gradient dilution after flow was increased with phenolamine and propranolol. Thus it was concluded that increasing flow would not have a large effect on glucose uptake. Accordingly, it was reasoned that vasodilators such as adenosine (114), bradykinin (123), sodium nitroprusside (115), and low doses of IGF-I (128), which increase total limb blood flow, would do little to influence glucose uptake in healthy subjects or improve insulin resistance. However, the reason that these vasodilators failed to influence glucose uptake may relate more to the fact that they preferentially increase flow into nonnutritive areas, where insulin-mediated glucose uptake is low (28, 118, 138). Indeed, all vasodilators may not act in the same way, and at least one other vasodilator may increase capillary recruitment. Thus, during a hyperinsulinemic euglycemic clamp, Baron et al. (14) used the endothelium-dependent vasodilator methacholine chloride to further increase flow and the NOS inhibitor L-NMMA to decrease flow. Whereas glucose uptake was increased by methacholine, it was reduced by L-NMMA. Taken together, the

data indicated a significant deviation from the Renkin equation for a fixed capillary surface area (141), indicating capillary recruitment. Furthermore, they examined whether the importance of flow depends on the degree of insulin sensitivity. They found that subjects with a higher glucose disposal rate (GDR) demonstrated a greater increase in leg glucose uptake due to methacholine infusion during the hyperinsulinemic euglycemic clamp, indicating that, in more-insulin-sensitive subjects, flow is more rate limiting for glucose uptake than in less-insulin-sensitive subjects.

Mather et al. (102) recently investigated the dependence of glucose uptake on flow in a group composed of lean, obese, and obese type 2 diabetic subjects. They found that, in the basal state, there was no difference in either GDR or leg blood flow. Moreover, when a GDR of $2,000 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$ was achieved in both a euglycemic hyperinsulinemic clamp and a hyperglycemic hyperinsulinemic clamp, subjects exhibited leg blood flows of 0.4 l/min regardless of their insulin sensitivity. Thus these data demonstrate the close coupling between flow and glucose uptake.

As indicated above, we have found capillary recruitment and glucose uptake to be closely linked in rats in vivo. Bulk flow was not significantly correlated with glucose uptake (137), but bulk flow increase may lead to an increase in capillary recruitment. However, it is yet to be finally resolved whether changes in glucose uptake result exclusively from changes in the latter rather than the former. There are definite situations where total flow can be increased in limbs in vivo, as with the vasodilators discussed above (114, 115, 123, 128) and epinephrine (136), without an increase in glucose uptake.

INSULIN RESISTANCE

Impaired increases in total flow. Insulin resistance is the state in which normally insulin-sensitive cells exhibit a low response to insulin.

The metabolic defects commonly demonstrated in vivo (reviewed in Ref. 15) are retained when muscle is biopsied from type 2 diabetic subjects and tested in vitro (43, 62) or when muscle from animal models with well-established insulin resistance is isolated and incubated (37). Thus metabolic abnormalities contribute significantly to the insulin resistance; however, this does not exclude the possibility that hemodynamic alterations also play a part in the decreased glucose uptake in vivo or have caused the metabolic and signaling derangements. If insulin-mediated increases in muscle blood flow and/or capillary recruitment can modulate glucose uptake in normal individuals, this raises the question whether these hemodynamic effects are blunted in states of insulin resistance and, second, whether this contributes to decreased glucose uptake in these subjects. In insulin-resistant subjects, basal limb blood flow is generally not altered (5, 40, 49, 94, 104, 172, 184, 194). However, there are studies demonstrating that insulin's ability to increase total flow is impaired in states of insulin resistance. Baron and

colleagues [Laakso et al. (94)] constructed dose-response curves for insulin-mediated increases in femoral blood flow and leg glucose uptake in healthy, obese, and obese type 2 diabetic subjects. Both dose curves were shifted to the right in the obese subjects, and this was even more pronounced in the type 2 diabetics. Furthermore, the type 2 diabetics also exhibited a decreased maximal response in both blood flow and glucose uptake; there was a significant correlation between leg blood flow and leg glucose uptake. Yet, not all studies agree with these findings, as a number of research groups have found no impairment of insulin-stimulated blood flow in type 2 diabetics (40, 172, 184). The explanation for some of the discrepancies may lie partly in the study design, as Laakso et al. clamped blood glucose levels to 4.5 mM, whereas Dela et al. (40) maintained plasma glucose levels at the ambient fasting concentration (5.8 mM for lean and 10.2 mM for type 2 diabetic subjects) (see also Table 1).

In general, it would seem that insulin is unable to increase blood flow in elderly subjects (60, 106). However, there is some controversy surrounding insulin resistance associated with other conditions. Impaired insulin-mediated increases in blood flow were seen in type 1 diabetics by Baron et al. (12) but not in other studies (197). Similarly, in hypertensive subjects, vasodilation was unaffected in some investigations (21, 116) but was blunted in others (11, 96). Baron et al. (11) found that the extent of insulin-mediated vasodilatation was inversely proportional to mean blood pressure, which then raises the question whether treatment aimed at lowering blood pressure would also improve insulin sensitivity. Indeed, this is often the case with drugs that act by facilitating vasodilation, since angiotensin-converting enzyme inhibitors (48, 167), α -blockers (3, 84, 166), and calcium antagonists (168) have all been shown to decrease insulin resistance. It is not known whether any of these agents alter blood flow distribution in muscle. Older, nonselective β -blockers that reduce peripheral blood flow tend to increase insulin resistance (54, 81).

Impaired capillary recruitment. Using 1-MX metabolism as an indicator of capillary recruitment, we have demonstrated that insulin-mediated changes in this parameter in anesthetized rats are indeed impaired in three states of acute insulin resistance. First, insulin resistance was induced by infusion of α -methylserotonin, a vasoconstrictor that has been shown to decrease nutritive capillary flow in the perfused rat hindlimb (117). In vivo, insulin's ability to increase total flow was abolished by α -methylserotonin, and the insulin-stimulated increase in 1-MX metabolism was decreased by 71% (137). These hemodynamic alterations by α -methylserotonin were associated with a 56% decrease in insulin-stimulated hindleg glucose uptake (Table 1). It has previously been shown that serotonin has no effect on glucose uptake in incubated muscles devoid of vascular involvement (138). Therefore, it was concluded that serotonin induced a hemodynamic insulin resistance by limiting insulin and glucose access to skeletal muscle. Because infusion of this vasocon-

strictor maintained an increase in blood pressure, this represented the first experimental model where hypertension and muscle insulin resistance could be seen to result from a single cause.

Second, insulin resistance was generated by acute infusion of TNF- α . Production of this cytokine is elevated in obesity, cancer, and sepsis (66, 71, 107, 124), conditions that also exhibit insulin resistance (122). After a 1-h infusion of TNF- α or saline, a 2-h insulin clamp was performed in anesthetized rats (201). TNF- α decreased glucose infusion rate (GIR), insulin-stimulated hindleg glucose uptake, and 2-deoxyglucose uptake into soleus and plantaris muscles. In conjunction with this, the increase in femoral blood flow and the increase in 1-MX metabolism due to insulin were prevented (Table 1). Alone, TNF- α had no effect on any of these parameters. It was proposed that TNF- α may inhibit the action of a vasodilatory molecule produced as a result of insulin's stimulation of glucose uptake. Alternatively, TNF- α may directly inhibit insulin's hemodynamic actions by impairing insulin signaling (201). In support of this, TNF- α has been reported to inhibit insulin signaling through the serine phosphorylation of IRS-1 (72). Furthermore, in aortic endothelial cells, TNF- α was shown to inhibit IRS-1 phosphorylation, PI 3-kinase activity, and phosphorylation of Akt/PKB and eNOS (89).

The third means of inducing insulin resistance was to infuse a combination of Intralipid and heparin. In a recent study, we have found that this combination decreased GIR, hindleg glucose uptake, and hindleg capillary recruitment during a physiological ($3 \text{ mU} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$) hyperinsulinemic euglycemic clamp (31). Bulk leg blood flow, measured at the femoral artery, was not affected (Table 1).

Insulin-mediated capillary recruitment in the Zucker obese rat is also impaired along with muscle glucose uptake, limb blood flow, and whole body glucose disposal (26) (Table 1).

Endothelial dysfunction and vascular complications of type 2 diabetes. Endothelial cells produce a variety of vasodilator and vasoconstrictor molecules that act on VSMC, thereby controlling vessel tone. In addition, it is now recognized that the endothelium also plays an important role in the regulation of vessel permeability and proliferation as well as blood fluidity and the adhesion of blood cells to the vascular wall (reviewed in Refs. 20, 174). Therefore, a range of complications can potentially result from a dysfunctional endothelium. This is of consequence, because it is also believed that endothelial dysfunction plays a key role in the development of the microvascular complications of type 2 diabetes, which include retinopathy, nephropathy, and neuropathy (reviewed in Ref. 95). Likewise, endothelial dysfunction is also central to the macrovascular complications of atherosclerosis and thrombosis, which lead to coronary artery, cerebrovascular, and peripheral vascular disease (95). The frequent association between endothelial dysfunction and insulin resistance, as well as with many of the features of the insulin resistance syndrome, including dyslipidemia,

hypertension, and hyperglycemia, has led to the proposal that endothelial dysfunction may be an important factor leading to insulin resistance (130, 174). Evidence in support of this theory comes from the fact that endothelial dysfunction precedes the development of type 2 diabetes (reviewed in Ref. 176). Furthermore, first-degree relatives of type 2 diabetics displayed alterations in micro- and macrocirculation despite normal glucose tolerance (19). One of the most important aspects of endothelial dysfunction is the impaired synthesis and/or increased degradation of NO (reviewed in Ref. 100), which has been suggested to result from increased oxidative stress (173). Because insulin is reported to increase flow and recruit capillaries through the production of NO (148, 165, 189), endothelial dysfunction is likely to contribute to the impaired vascular actions of insulin in states of insulin resistance.

It is also likely that, once established, the features of the insulin resistance syndrome act to exacerbate endothelial dysfunction. Alone, hyperglycemia, dyslipidemia, and hypertension have all been shown to lead to endothelial dysfunction, even in the absence of insulin resistance (177). Although not essential for the development of endothelial dysfunction, hyperglycemia could be considered one of the most important factors contributing to diabetic complications (reviewed in Ref. 58). Hyperglycemia is thought to lead to the generation of reactive oxygen species, partly through the autooxidation of glucose and increased activity of the sorbitol pathway (175). Also, when in excess, glucose glycosylates proteins, and these products can then rearrange to form advanced glycation end products, which quench NO and are involved in the formation of atherosclerotic lesions (56).

Interstitial insulin levels. There is a delay in the onset of insulin action in vivo compared with that in vitro (129, 157, 196), which may be due to transendothelial insulin transport. In earlier in vitro studies, King and Johnson (91) demonstrated a receptor-mediated transport of insulin across endothelial cells. However, more recent studies conducted in vivo dispute these findings, claiming that insulin moves across the endothelium predominantly via diffusion, since the process did not become saturated (163).

Decreased transport of insulin across the endothelium would be expected to be reflected in interstitial insulin concentrations lower than those in plasma. In 1989, Yang et al. (196) measured lymph insulin in dogs as an estimate of the interstitial level and found that the ratio of insulin concentration in plasma to that in lymph was in fact 3:2 in the basal state, during a hyperinsulinemic euglycemic clamp, and in the period after insulin infusion ceased. This work has been confirmed by microdialysis studies in both animals (68) and humans (158). Again, the interstitial levels were 30–50% lower than those in plasma, suggesting that the endothelium does form a barrier for insulin access to muscle.

It was noted that the time course of insulin-mediated glucose uptake more closely followed the rise in inter-

stitial insulin than the rise in plasma insulin (196). This raised the possibility that, in states of insulin resistance, insulin delivery and/or transport across the endothelium may be limiting. This would be consistent with the findings that, in obese subjects, there is a delay in the rate of activation of insulin-stimulated glucose disposal (132). However, when interstitial insulin and glucose concentrations were examined in insulin-resistant subjects, no differences were found between control and type 2 diabetic subjects during hyperinsulinemia (158). Moreover, there was no difference in the time course of appearance of insulin in the interstitial fluid between the two groups (33). This led the authors to conclude that insulin access is not limiting to glucose uptake in type 2 diabetes. Similarly, there was no difference in the interstitial glucose concentration during the clamp between the groups; however, Cline et al. (33) reported interstitial values equal to arterial levels, whereas Sjöstrand et al. (159) found the glucose concentration to be lower in interstitial than in arterial fluid. Although these results appear to be evidence against a hemodynamic component to insulin resistance, it must be remembered that the interstitial insulin levels are quite low and the microdialysis probe recoveries are very low, so that it is possible that the technique is not sensitive enough to detect small differences between the two groups. In addition, it is unknown what effect changes in capillary recruitment now known to be mediated by insulin itself have on the average interstitial concentration of insulin. Recent perfused hindlimb studies (120) indicate that this may be marked.

EXERCISE ENHANCEMENT OF INSULIN SENSITIVITY

Muscle contraction increases total blood flow to muscle (74), recruits capillaries (70), and stimulates the translocation of GLUT4 to the plasma membrane, thereby increasing glucose uptake (44). Exercise-stimulated glucose uptake and maximal insulin-mediated glucose uptake are additive (142), and two distinct signaling pathways appear likely. Exercise-mediated glucose uptake, unlike insulin-mediated glucose uptake, is not inhibited by wortmannin and may involve AMP kinase rather than signaling through PI 3-kinase (146). NOS inhibition has been reported to inhibit exercise-mediated glucose uptake in humans (18) but not in mice (143). It remains to be tested whether blood flow redistribution occurs in muscle as a result of eNOS inhibition when exercise is the stimulus for glucose uptake. For insulin, the reported effect of NOS inhibition to partly inhibit insulin-mediated glucose uptake is attributed to vascular effects in both humans (8) and rats (189).

It is well established that prolonged exercise leads to enhanced insulin sensitivity and maximal insulin responsiveness (17). Evidence for this comes from comparisons of trained athletes with untrained subjects (46) as well as from training intervention studies. Glucose uptake measured during a hyperinsulinemic euglycemic clamp in individuals before and after 6 wk of

endurance training revealed a 30% increase in glucose uptake (161). Endurance training leads to increased cardiac output and muscle oxygen extraction, resulting in a higher maximal oxygen uptake. In addition, a number of metabolic and structural changes occur in muscle. Skeletal muscle's ability to oxidize pyruvate, fatty acids, and ketones is increased as a result of elevated levels of mitochondrial citric acid cycle enzymes as well as enzymes involved in fatty acid breakdown and ketone utilization (reviewed in Ref. 67). Apart from hexokinase activity, which is significantly increased, changes in glycolytic enzymes are generally small. Specific alterations differ depending on the muscle fiber type, and often no differences are seen overall (reviewed in Ref. 67). It has also been reported that there is no change in glycogen synthase activity (24). In accordance with these changes in enzyme profiles, there is generally a shift from fast type II to slow type I fibers following exercise training (reviewed in Ref. 38).

The augmented insulin sensitivity may also be due to alterations in insulin-signaling molecules. In fact, raised mRNA levels of the insulin receptor, IRS-1, and MAP kinase (ERK1) have been reported in exercise-trained rats (90). In addition, other studies have found increases in IRS-1- and IRS-2-associated PI 3-kinase activity and Akt phosphorylation (24). Possibly one of the most important changes is the elevated GLUT4 protein content following exercise training in healthy humans (73).

It is also possible that hemodynamic factors may be involved in the elevated insulin sensitivity following exercise (77). As mentioned previously, acute exercise increases total flow and capillary recruitment. Moreover, some studies have found elevated basal blood flow in athletes compared with untrained individuals (46); however, this is not always significantly increased (59). Nevertheless, Hardin et al. (59) did report that insulin-stimulated blood flow was 31% higher in athletes than in controls. Furthermore, Dela et al. (41) found that the insulin-stimulated blood flow was increased after 10 wk of one-legged training in both healthy subjects and type 2 diabetic patients. Although there are studies that have not observed differences in capillarization (46), it has been reported by others that there are increases in capillary density in skeletal muscle following training in humans (1, 63) and in rats (57). If this is the case, the increased capillary surface area may provide a greater scope for insulin-mediated capillary recruitment. It is also possible that exercise training may enhance insulin's ability to increase total flow and/or capillary recruitment, thereby contributing to the enhanced glucose uptake.

Even though interpretation may be complicated by the effects of aging, exercise-stimulated glucose uptake is generally not impaired in type 2 diabetics (36, 52). Consequently, there are several reports that exercise is beneficial in both the prevention and treatment of type 2 diabetes (53, 87, 127). However, some studies showed either no or only minimal beneficial effects in the treatment of diabetes (17, 149). The reason for this may be due to the duration of the disease, the training

protocol, or the method to determine insulin sensitivity. Nevertheless, there are studies that report weight loss and favorable effects on lipid metabolism, leading to increases in HDL cholesterol and decreases in LDL cholesterol (87).

At the experimental animal model level, voluntary exercise training was found to have a marked improvement in insulin responses under hyperinsulinemic euglycemic conditions. Whole body GIR, hindleg glucose uptake, and capillary recruitment (1-MX disappearance) were each increased compared with untrained animals as a result of the training period in which the animals had run an average of 18 km (139) (Table 1).

CONCLUSION

New methods have shown that a major hemodynamic effect of insulin is to increase nutritive capillary blood flow in muscle. Current evidence suggests that this effect is independent of an increase in bulk flow to muscle that often accompanies insulin's action. Insulin-mediated capillary recruitment may have similar beneficial effects to those of exercise relating to hormone and substrate delivery to the muscle cells. Interventions that impair capillary recruitment give rise to insulin resistance with decreased insulin-mediated glucose uptake in muscle. Conversely, exercise training enhances both insulin-mediated capillary recruitment and muscle glucose uptake.

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